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CONTENTS OF THIS ISSUE

Antiseptics of the Quarternary Ammonium Type in the Presence of Positive and Negative Gelatin 201
L. F. Tice and R. Pressman

Characterization of the Glycosidal Principle of *Viburnum Prunifolium* 205
Harry K. Iwamoto, William E. Evans, Jr., and John C. Krantz, Jr.

A Note on the Pharmacology of *Viburnum Purnifolium* and Its Glycoside 207
William E. Evans, Jr., Harry K. Iwamoto, and John C. Krantz, Jr.

The Spectrophotometric Assay of Hexestrol and Some of Its Pharmaceutical Preparations 209
Hartmann Goetze and L. D. Seif

The White Rat in the Biological Assay of Senna and Its Preparations 211
Hugh C. Vincent, C. H. Waldon, and C. O. Lee

Spirit of Chloroform, N. F. VII 213
Samuel W. Goldstein

A Chemical Study of Aromatic Spirit of Ammonia 216
Charles F. Poe and Martin E. Hultquist

Hydrogenated Castor Oil as an Ointment Base. VII. Sulfated Product in Antiseptic Ointments 218
George W. Fiero and Ted A. Loomis

Evaluation of Some Liquid Antiseptics . . . 219
Alvin R. Whitehill

Hexadienol, A Locally Acting Diaphoretic and A New Diagnostic Agent 221
Theodore Koppanyi

Book Reviews . . . . . 208 and 224

Abstracts 201–224

Advertising—Cover pages 2, 3, 4 and pp. II to VIII

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Antiseptics of the Quaternary Ammonium Type in the Presence of Positive and Negative Gelatin*

By L. F. Tice and R. Pressman

Within the past few years considerable interest has been aroused in the quaternary ammonium type of antiseptic. Although previously reported in the literature, the work of Domagk (1) stimulated research on these compounds. A number of commercial products of this type are already marketed, e.g. "Zephiran Chloride" (a mixture of high molecular, alkyl-dimethyl-benzyl-ammonium chlorides), "Phemerol" (*para-tertiary*-octyl phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride) and "Ceepryn" (cetyl-pyridinium chloride). Antiseptics of this type differ from the ordinary kind in many respects which have been widely described. Their bactericidal efficiency seems to be related to some degree to their surface activity coupled with the fact that they are cation-active in contrast to most wetting agents and detergents which are anion-active or anionic. The theory has been advanced (2, 3) that they in some manner interfere with the membrane equilibria essential for the normal metabolism of the microorganism, resulting in either its destruction or inhibiting its reproduction. The presence of anionic substances has been found in many instances to impair greatly the bactericidal efficiency of these quaternary ammonium compounds due undoubtedly to the opposite charge on the two substances. Thus soap is incompatible with the cationic antiseptics and its use preceding their application requires thorough washing with water before these antiseptics are applied (4).

Gelatin has been reported (5) as incompatible with one of these antiseptics ("Ceepryn") and since gelatin might be used in preparing suppositories or other types of medication involving the quaternary ammonium compounds this investigation was begun. Its object was to study the behavior of various gelatins with these cationic antiseptics from the physicochemical standpoint and to determine the effect of gelatin on their bactericidal efficiency.

That there exist two entirely different types of gelatin has been clearly established (6). This fact is recognized in the official definition for gelatin given in the U. S. P. XII (7) which states "Gelatin derived from an acid-treated precursor exhibits an isoelectric point between *p*H 7 and *p*H 9, while Gelatin derived from an alkali-treated precursor has an isoelectric point between *p*H 4.7 and *p*H 5.0." It is for this reason that the two gelatins themselves are incompatible between the approximate *p*H limits of 5 and

* Received Dec. 31, 1944, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

7 since they are oppositely charged, producing extreme turbidity (8). This behavior has been found by one of us (L. F. T.) to be due to coacervate formation between the oppositely charged gelatin micelles.

Between the *p*H limits of 5 and 7 it is therefore possible to have a gelatin which behaves as an anionic agent or as a cationic agent depending upon its isoelectric point, which in turn depends upon the treatment given its precursor during manufacture. For this investigation Pharmagel A was employed as a gelatin obtained from an acid-treated precursor and Pharmagel B as a product derived from an alkali-treated precursor. Pure samples of both "Phemerol" and "Ceepryn" were obtained, as well as an aqueous solution (12.8 per cent) of "Zephiran Chloride." It was quite important that pure materials without foreign electrolyte be used as will be seen later.

Preliminary experiments gave definite evidence of incompatibility between the cationic antiseptics and gelatin, but only under those conditions wherein the gelatin was anionic. Since this incompatibility was suspected to be due to coacervation, experiments were undertaken to determine if such was the case. DeJong and his students (9) who did the classic work on coacervation have described these phenomena quite well for a number of colloid systems. Briefly it is the result of the attraction between oppositely charged micelles or ions resulting in a "swarm" with changes being produced in solvation and therefore turbidity, viscosity and other properties.

The coacervate is in most instances dispersed by added electrolyte with the removal of the state of desolvation and a return to normal or near normal viscosity and clarity. Furthermore, a definite Schulze-Hardy effect is noticed in that the valence of the ions of the added electrolyte has a quantitative influence. In those instances where charge is a function of the *p*H there is likewise a shift in the turbidity maximum when relative turbidity is plotted against the per cent of this component.

EXPERIMENTAL

Experimental evidence was obtained showing that all three antiseptics, "Zephiran," "Ceepryn," and "Phemerol" behaved in an analogous manner, in so far as gelatin was concerned. "Phemerol" was then chosen for the detailed experiments since it was available in crystalline form and in large quantities. A 4% solution of both A and B gelatins was prepared and each adjusted to *p*H 6.0. A 2% solution of Phemerol was made and then two series of tubes were prepared, one series for each type of gelatin, in which the ratio of gelatin to Phemerol was varied, the tubes carefully shaken and then allowed to stand at room temperature for several hours. During this time turbidity developed in certain tubes going through a maximum as indicated in Table I.

From the results shown in Table I a number of observations were made. It was obvious that only a negative or an anionic gelatin produced turbidity with the cationic antiseptic and that this only took place in certain ratios passing through a maximum.

When neutral salts were added to turbid mixtures of negative gelatin and the cationic antiseptics, clarity was restored. This gave evidence of its being a coacervation phenomenon so the effect of valence was studied. Using 10-cc. portions of a coacervate mixture containing negative gelatin and cationic antiseptic as above, various amounts of sodium chloride, calcium chloride, aluminum chloride and sodium sulfate were added. The minimal molar concentration of each required to bring about complete clarity follows:

Salt	Molar Concentration
NaCl	0.12
$CaCl_2$	0.04
$AlCl_3$	0.002
Na_2SO_4	0.06

Thus the Schulze-Hardy effect was observed, namely that $Al^{+++} > Ca^{++} > Na^{+}$ and $SO_4^{--} > Cl^{-}$ in their peptizing action.

Viscosity measurements with gelatin as a means of detecting changes in solvation are not as readily performed as measurements of gel strength using a Bloom Gelometer. The following samples were prepared, aged for eighteen hours in a constant temperature bath at 10° C., and the gel strength was determined by a modified Bloom Gelometer constructed for "jelly powder" control.

	Composition	Jelly Strength, Gm.
No. 1	105 cc. 4% A (*p*H 6.0), 45 cc. 2% P	87.5
No. 2	105 cc. 4% B (*p*H 6.0), 45 cc. 2% P	19.0
No. 3	105 cc. 4% B (*p*H 6.0), 45 cc. 2% P + 1.25 Gm. NaCl	64.0
No. 4	105 cc. 4% A (*p*H 6.0), 45 cc. water (Control)	93.5
No. 5	105 cc. 4% B (*p*H 6.0), 45 cc. water (Control)	75.0
No. 6	105 cc. 4% A (*p*H 6.0), 45 cc. water + 1.25 Gm. NaCl	90.0

These results were quite significant and they further verified coacervate formation between anionic gelatin and the cationic antiseptics. For example, "Phemerol" with positive gelatin (No. 1) only slightly lowered the gel strength from 93.5 Gm. (No. 4 Control) to 87.5 Gm. With negative gelatin (No. 2) a reading of only 19 Gm. was obtained as compared with the control (No. 5) of 75 Gm. Sodium Chloride alone was shown (No. 6) to effect gel strength but little, but when added in a mixture otherwise exhibiting coacervation it almost restored it to a normal condition of solvation. Thus No. 2 without added salt gave a reading of 19 Gm. and with added salt a reading of 64 Gm. (No. 3).

As a final check on the validity of the theory of coacervate formation in this system, the *p*H of the gelatin was adjusted to 8.0 in order to determine if the ratio of negative gelatin to "Phemerol" producing

maximum turbidity would be shifted. This would be expected, since by an upward shift in *p*H the gelatin would be further removed from its isoelectric point and would therefore carry a higher negative charge. Preliminary experiments indicated that the gelatin-Phemerol ratio would have to be varied within more narrow limits than in Table I in order to obtain the exact maximum reading.

TABLE I[a]

PA_0	10 cc. A + 0 cc. P	Clear
PA_1	9 cc. A + 1 cc. P	Clear
PA_2	8 cc. A + 2 cc. P	Clear
PA_3	7 cc. A + 3 cc. P	Clear
PA_4	6 cc. A + 4 cc. P	Clear
PA_5	5 cc. A + 5 cc. P	Clear
PA_6	4 cc. A + 6 cc. P	Clear
PA_7	3 cc. A + 7 cc. P	Clear
PA_8	2 cc. A + 8 cc. P	Clear
PA_9	1 cc. A + 9 cc. P	Clear
PA_{10}	0 cc. A + 10 cc. P	Clear
PB_0	10 cc. B + 0 cc. P	Clear
PB_1	9 cc. B + 1 cc. P	Slightly turbid
PB_2	8 cc. B + 2 cc. P	Turbid
PB_3	7 cc. B + 3 cc. P	Very turbid
PB_4	6 cc. B + 4 cc. P	Very turbid
PB_5	5 cc B + 5 cc. P	Slightly turbid
PB_6	4 cc. B + 6 cc. P	Clear
PB_7	3 cc. B + 7 cc. P	Clear
PB_8	2 cc. B + 8 cc. P	Clear
PB_9	1 cc. B + 9 cc. P	Clear
PB_{10}	0 cc. B + 10 cc. P	Clear

[a] A = 4% Pharmagel A (*p*H 6.0); B = 4% Pharmagel B (*p*H 6.0); P = 2% Phemerol (when 2% Phemerol was replaced with Zephiran Chloride or Ceepryn, an identical series was obtained).

A series similar to that in Table I was prepared at both *p*H 6.0 and *p*H 8.0 with the quantities of each component (B and P) varying by only 0.1 cc. in each tube. The tube exhibiting maximum turbidity in each series was determined by passing a beam of light through the tube and measuring in foot-candles the emergent beam. A graph illustrating this maximum at each *p*H level is shown in Fig. 1.

A definite shift in the ratio producing maximum turbidity was observed, a characteristic behavior of coacervate systems.

Bacteriological Data. Since the existence of coacervation between the cationic antiseptics and negative gelatin had been observed, it became of interest to determine whether this incompatibility reduced the bactericidal efficiency of these antiseptics and whether any difference could be shown in the effect of positive and negative gelatins. Since positive gelatins are compatible in all ratios and negative gelatins are not, it was conceivable that some difference might be demonstrable.

Solutions were prepared as follows:

P	2% Phemerol (Control)
PA	350 cc. 4% Pharmagel A (*p*H 6.0) 150 cc. 2% Phemerol
PB	350 cc. 4% Pharmagel B (*p*H 6.0) 150 cc. 2% Phemerol
PB + Salt	350 cc. 4% Pharmagel B (*p*H 6.0) 150 cc. 2% Phemerol 5 Gm. NaCl

The bactericidal efficiency of these combinations was tested first by the phenol coefficient test according to the F. D. A. method (10). The results obtained by this method were too inconsistent to warrant any interpretation.

In an attempt to overcome this inconsistency the

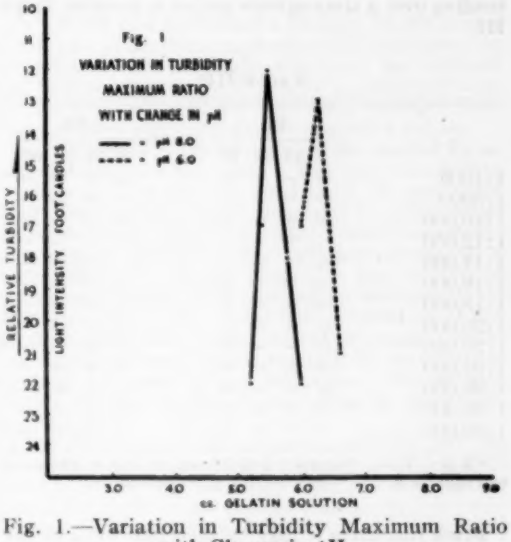

Fig. 1.—Variation in Turbidity Maximum Ratio with Change in *p*H.

TABLE II[a]

	P				PA				PB				PBS			
	Minutes															
	5	15	30	60	5	15	30	60	5	15	30	60	5	15	30	60
1:1000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1:5000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1:10,000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1:12,000	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
1:14,000	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1:16,000	+	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−
1:18,000	+	+	+	−	+	−	−	−	−	−	−	+	+	+	−	−
1:20,000	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−
1:22,000	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−
1:24,000	+	+	−	−	+	−	−	−	−	−	+	−	+	+	−	−
1:26,000	+	+	−	−	+	−	−	−	+	−	−	−	+	+	−	−
1:28,000	+	+	+	−	+	+	−	−	+	−	−	−	+	+	−	−
1:30,000	+	+	+	+	+	+	−	−	+	−	−	−	+	+	−	−

[a] Key: − = no growth; + = growth; P = Control (Phemerol); PA = Phemerol + Pharmagel A; PB = Phemerol + Pharmagel B; PBS = Phemerol + Pharmagel B + NaCl.

technique was slightly modified in respect to size of the transfer and the time intervals. The test organism was Staphylococcus aureus 209 grown in F. D. A. nutrient broth. To 5 cc. of diluted sample at 20° C. was added 0.5 cc. of an eighteen to twenty-four hour growth of the culture. After intervals of five, fifteen, thirty, and sixty minutes 0.05 cc. of the mixture was transferred to 10 cc. of sterile nutrient broth. Subcultures were incubated at 37° C. for forty-eight hours. Tubes which failed to show growth after forty-eight hours were further tested for bacteriostatic activity by transferring 0.05 cc. of the culture to a fresh tube of medium. Although the modified technic produced better results, they were not always consistent.

A typical experiment which was extended over a sixty-minute period and one illustrating some inconsistency is given in Table II.

It will be noted that no marked difference in the efficiency of the Phemerol was observed when either positive or negative gelatin was present.

Since the dilutions in these experiments became great enough to reduce markedly the gelatin content, another series of experiments was performed in which a 1% solution of each gelatin at a *p*H of 6.0 was used as a diluent. A typical experiment extending over a sixty-minute period is given in Table III.

TABLE III[a]

	PA				Minutes	PB			
	5	15	30	60		5	15	30	60
1:1000	−	−	−	−		−	−	−	−
1:5000	−	−	−	−		−	−	−	−
1:10,000	−	−	−	−		−	−	−	−
1:12,000	−	−	−	−		−	−	−	−
1:14,000	−	−	−	−		−	−	−	−
1:16,000	−	−	−	−		+	−	−	−
1:18,000	−	−	−	−		−	−	−	−
1:20,000	−	−	−	−		−	−	−	−
1:22,000	−	−	−	−		−	+	−	−
1:24,000	−	−	−	−		−	+	−	−
1:26,000	−	−	−	−		−	−	−	−
1:28,000	−	−	−	−		−	−	−	−
1:30,000	−	−	−	−		−	−	−	−

[a] Key: PA = Phemerol and Gelatin A; PB = Phemerol and Gelatin B; − = no growth; + = growth.

From this table it may be seen that even in the presence of 1% gelatin there is no reduction in bactericidal efficiency; indeed there seems to be an increase in efficiency.

Thus the bacteriological investigations gave results indicating that the efficiency of Phemerol is not reduced by either type of gelatin even in the presence of coacervation. It is true that there were inconsistencies in the results: (*a*) in the concentration of the same preparation which would kill, say, a standard culture of S. aureus in fifteen minutes but not in five minutes, and (*b*) the variations in efficiencies from day to day between the different preparations. It is noteworthy, however, that the control was at no time more efficient than any of the combinations used. For example, in 9 experiments the following results were obtained:

PB > P > PA = PBS
PB > P > PBS = PA
PBS = P > PA > PB
PBS = PA = P > PB
PB > PA > PBS > P

In the other four tests the values were the same, i.e., P = PA = PB = PBS.

Reports from other laboratories also indicate a lack of uniformity in results (11, 12).

SUMMARY AND CONCLUSIONS

1. The behavior of positive and negative gelatins with a number of quaternary ammonium antiseptics has been studied.

2. Evidence is presented that negative gelatins are capable of producing coacervates with these cationic antiseptics in certain ratios.

3. No coacervate is produced by a positive gelatin in any ratio.

4. The bactericidal efficiency of "Phemerol" is not reduced in the presence of either type of gelatin even when coacervation exists.

5. There is a possibility that gelatin augments the bactericidal efficiency of "Phemerol.

6. Bactericidal efficiency tests with the quaternary ammonium compounds yield results that are rarely reproducible. This phenomenon is under further study.

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Characterization of the Glycosidal Principle of *Viburnum Prunifolium**

By Harry K. Iwamoto, William E. Evans, Jr., and John C. Krantz, Jr.

Within the past few years, preparations of pharmacognostically authentic *Viburnum prunifolium* have been shown to possess sedative effect on the isolated uterus and uterus *in situ*, both on animals and human subjects (1–4).

Since the inception of the chemical studies of the constituents of the Viburnums, the presence of a glycosidal substance has been either hinted or reported (5–9). In 1942, Evans, Harne and Krantz (3) described the isolation of a glycosidal principle in crystalline form from an authentic sample of *Viburnum prunifolium*, which produced the characteristic relaxation of uterine strips.

In a recent report (10), a refinement of technique in the method of isolation of white, crystalline glycosidic material is described. It was possible to obtain a quantity sufficient to carry out chemical investigation on the nature of the glycoside.

Through the performance of fundamental tests and reactions, preparation of a derivative and comparative studies, the uterine principle isolated from authentic *Viburnum prunifolium* was found to be identical with the glucoside, salicin.

EXPERIMENTAL

Elementary Analysis.—The compound gave the following results when analyzed[1] for carbon and hydrogen: weight of sample, 4.380 mg. and 3.455 mg.; weight of CO_2, 8.77 mg. and 6.91 mg.; weight of H_2O, 2.63 mg. and 2.05 mg.; hence per cent of hydrogen, 6.72 and 6.64; per cent of carbon, 54.64 and 54.58.

The same glycoside was examined for other elements and found to contain no nitrogen, halogen, sulfur, and phosphorus. Presumably the remaining element was oxygen. Working on this hypothesis, formulation of $C_{13}H_{20}O_8$ was developed as the empirical formula, approximating the value obtained in the molecular-weight determination as closely as possible.

Molecular-Weight Determination.—Employing the cryoscopic method, molecular weights of 232, 234, and 226 were calculated from the data obtained as follows: weight of sample, 1.500 Gm., 0.400 Gm., and 0.400 Gm.; volume of solvent (water) used, 60.0 cc., 15.0 cc., and 15.0 cc.; freezing point depression, 0.200° C., 0.212° C., and 0.219° C.

Physical Constants.—The glycoside, once recrystallized from water, melted sharply at 200–201° C. (corr.). Half of a gram of the material dissolved completely in 11.5 cc. of water at room temperature. The water solution was extremely bitter to the taste and neutral to litmus paper. The glycoside was soluble in alcohol but not in ether.

An aqueous solution containing 0.4470 Gm. of crystalline glycoside, made up to 100 cc. with distilled water, rotated 0.55 circular degree to the left, in a 2-dm. tube, using sodium light, at 25° ± 1°; hence $[\alpha]_D^{25} = -61.6$.

CLASS REACTIONS

1. Molisch Test.—A sample of the substance consistently gave a positive violet coloration to the Molisch reagent, suggesting the presence of a carbohydrate.

2. Fehling's Test.—An aqueous solution of the material failed to reduce Fehling's solution. Reduction occurred only after acid hydrolysis. This indicated that the original product was complete acetal or glycoside.

3. Bial's and Seliwanoff's Tests.—To determine the nature of the reducing sugars obtained after acid hydrolysis of the glycoside, Bial's test for pentoses and Seliwanoff's test for ketose were performed both on the glycoside and on the sugar residue. The sugar portion was neither a pentose nor a ketose.

4. Pyrolysis.—The glycoside was charred to an amber color in a test tube. The odor was highly reminiscent but difficult to define; it was found later that salicylaldehyde possesses a similar odor. A few drops of $FeCl_3$, T. S., when added to an aqueous solution of the pyrolytic products produced a violet color. With conc. H_2SO_4, a few crystals of the glycoside gave a red color which disappeared on dilution with water. A chloroform solution of the products of pyrolysis gave no red or orange coloration to Friedel and Crafts' reaction for an aromatic nucleus, but gave a tan coloration. Friedel and Crafts' reaction on the original substance gave similar results.

5. Hydrolysis.—An aqueous solution of the glycoside was gently boiled with about 2–5% HCl until a white, oily turbidity resulted. On further heating, a light brown colored resinous product was observed on the surface of the liquid which solidified on cooling. The resin was filtered off and pulverized to a tan-colored powder when dry. It was soluble in alcohol and ether, and insoluble in water. The filtrate was subjected to a test for osazone formation and a qualitative test for mannose.

(*a*) *Osazone Formation.*—Yellow osazone formed within five minutes of heating in a boiling water bath. The osazone, purified from water-alcohol, melted at 203° C. The mixed melting point of the osazone with a freshly prepared glucosazone (m. p. 204° C.) gave no indication of depression.

(*b*) *Qualitative Test for Mannose.*—In a former communication (3), the glycoside was thought to contain mannose. The sugar residue of the filtrate was examined for mannose according to the method of Denn, Jackson, and Ballard (11) using codeine-H_2SO_4 and heroine-H_2SO_4 reagents. Mannose was concluded to be absent.

The tan-colored powder which was obtained in the acid hydrolysis was tested in the following manner:

(*a*) *Melting Point.*—The crude material began to shrink at 125° C., liquefied at 140° C. and melted

* Received Feb. 21, 1945, from the Dept. of Pharmacology School of Medicine, University of Maryland, Baltimore, Md.

[1] Carbon and hydrogen analysis was made by Arlington Laboratories, Fairfax, Va.

with effervescence at 157–158° C. At a temperature above 185° C., the light-colored melt turned very dark. The crude material was dissolved in dilute NaOH solution and gently boiled with activated charcoal. The solution had darkened; the aglycone must have been of phenolic nature. On cooling and acidification with dilute HCl, a flocculent tan-colored precipitate resulted. When dry, the product began to shrink at 125° C., turned brown at 130° C., and melted at 180° C.

(*b*) *Hydroxyl Group.*—The dioxane solution containing the aglycone gave a reddish brown precipitate when ceric nitrate reagent (12) was added dropwise. The presence of either the phenolic or enolic hydroxyl group was indicated. A hydroalcoholic solution of the aglycone gave varying results to $FeCl_3$, T. S. Some samples gave a faint green coloration, others gave golden-yellow color not unlike the color of the reagent. This anomalous behavior was due to difference in the degree of polymerization of the aglycone. The Theis and Benedict test for phenols (13) was positive; ethylacetoacetate similarly gave a positive result.

(*c*) *Ene-linkage.*—Because of the indications that either a phenolic or enolic hydroxyl group must be present in the structure, bromine water solution was added dropwise into the hydroalcoholic solution of the aglycone. The bromine was rapidly decolorized with the formation of canary-yellow colored precipitate. A few drops of bromine in carbon tetrachloride solution was added into absolute alcohol solution of the aglycone. After shaking, a stirring rod dipped in conc. NH_4OH, placed over the container resulted in white fumes. Potassium permanganate solution was promptly decolorized by hydroalcoholic solution of the aglycone; control with alcohol-water was run simultaneously.

(*d*) *Aromatic Nucleus.*—A chloroform solution of the aglycone was poured into freshly sublimed aluminum chloride. Several condensations were performed but none of the results gave orange, red, green, or purple colors. The results obtained were sometimes flesh color and sometimes faint yellow-orange color.

6. Emulsin Cleavage.—A small sample of the glycoside was dissolved in distilled water and about an equal quantity of emulsin added. The mixture was placed at room temperature for twenty-four hours. A portion was filtered and tested with Fehling's solution; reduction took place in thirty seconds. To another filtered portion a few drops of $FeCl_3$, T. S., were added; immediate violet coloration resulted.

The products of enzymatic cleavage indicated that the glycosidic linkage was β, and that the aglycone was phenolic in nature. Since no heat was involved in the cleavage, it suggested that on heating, the simple aglycone (which responds to $FeCl_3$, T. S.) polymerizes to a complex aglycone (which no longer responds to $FeCl_3$, T. S.). To test this hypothesis adequately, degradative acid hydrolysis of the glycoside was undertaken.

7. Degradative Acid Hydrolysis.—If during the hydrolysis of the glycoside, a simple aglycone was formed, this should be detected some time during the course of the hydrolysis as soon as the presence of reducing sugar becomes evident. On continued heating, the aglycone should begin to polymerize and ultimately fail to give positive test to $FeCl_3$, T. S.

This was found to be true as the data indicate in Table I. The solution was prepared by dissolving 0.4 Gm. of the glycoside in 25 cc. of distilled water and adding 4 drops of conc. HCl. At the first sign of turbidity, a sample was removed and evaporated to dryness on a water bath. The residue, when taken up in hydroalcohol, gave no positive coloration to $FeCl_3$, T. S.

TABLE I.—HYDROLYSIS OF GLYCOSIDE

Time of Heating, Min.	Color of Fehling's Solution[a]	Color of $FeCl_3$, T. S.	Appearance of Solution
0			Clear, water-white
3	No reduction	Yellow	Clear, water-white
9	Slight reduction	Orange-yellow	Clear, water-white
11	Red ppt. +	Yellow-green	Clear, water-white
14	Red ppt. ++	Pale green +	Clear, water-white
17	Red ppt. +++	Pale green ++	Clear, water-white
19	Red ppt. ++++	Pale green +++	Clear, water-white
21	Red ppt. +++++	Pale green ++++	Clear, water-white
29	Red ppt. ++++++	Pale green +++++	Clear, water-white
40	Orange-red ppt.	Dark olive-green	First sign of turbidity

[a] + indicates intensity of precipitate or color.

TABLE II. COMPARATIVE STUDY OF SALICIN AND VIBURNUM PRINCIPLE

	Melting Point, °C. (corr.)
Viburnum principle	199–200, 200–201
Salicin (Merck)	200–201
Mixed melting point, 50% Viburnum principle and 50% salicin	200–201
Mixed melting point, 75% Viburnum principle and 25% salicin	200
Penta-acetyl derivative of Viburnum principle	129
Penta-acetyl salicin	130
Mixed melting point, 50% penta-acetyl Viburnum principle and 50% penta-acetyl salicin	129–130
Mixed melting point, 75% penta-acetyl Viburnum principle and 25% penta-acetyl salicin	129–130

The Preparation of a Derivative and Comparative Study.—On the basis of the tentative empirical formula, $C_{13}H_{20}O_7$, 6 carbon atoms were allotted to the sugar moiety and 7 carbon atoms to the aglycone portion. It occurred that since aglycones are usually biologically active, the structure that most likely will cause smooth muscle relaxant action would be benzyl alcohol. A $-CH_2OH$ group on the side chain would fit in perfectly using up the last of the 7 oxygen atoms in the empirical formula.

The crystalline glycosidal principle of *Viburnum prunifolium* melted exactly at the temperature where the glucoside, salicin, melts. The Viburnum principle was studied comparatively with salicin and data obtained as indicated in Table II.

SUMMARY

The crystalline glycosidal principle obtained from authentic *Viburnum prunifolium* was found to be chemically identical with the glucoside, salicin, as shown above in the experimental data.

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A Note on the Pharmacology of *Viburnum Prunifolium* and Its Glycoside*

By William E. Evans, Jr., Harry K. Iwamoto, and John C. Krantz, Jr.

In former studies, Evans, Harne, and Krantz (1) reported the isolation of a glycoside from the pharmacognostically authenticated bark of *Viburnum prunifolium* N. F. The glycoside was shown to be active in relaxing the rat's uterus *in vitro* and also relaxing human uterine strips under the same conditions. The method of extraction employed at that time yielded a product which was amorphous and exceedingly hygroscopic. Since that time the present authors have modified the extraction process and succeeded in obtaining invariably a crystalline glycoside.

The purpose of this communication is to set forth the procedure for the extraction of the glycoside in crystalline form and to correlate its activity with that of viburnum. Elsewhere (2) we have shown it to be identical with salicin obtained from the poplar and willow barks.

EXPERIMENTAL

Isolation of the Crystalline Glycoside.—*Viburnum prunifolium*, coarsely comminuted, is percolated to exhaustion with alcohol. The alcoholic percolate is concentrated *in vacuo* at a temperature of 60° C. to the consistency of a thin syrup. The syrup is extracted repeatedly with water discarding the water-insoluble residue. To the combined aqueous extractions, alcohol is added to effect a concentration of approximately 80%. Resins, tannins, and sugar are further precipitated by the addition of an aqueous saturated solution of barium hydroxide. The precipitate is removed by filtration. From the filtrate the excess of barium is precipitated by the addition of diluted sulfuric acid until the *p*H of the mixture lies between 6.8 and 7.0. If necessary, sodium sulfate may be added to precipitate completely the barium. The mixture is filtered clear and evaporated at 60° C. *in vacuo* to the consistency of a thick syrup maintaining the foregoing *p*H range during the process of evaporation. This thick syrup is extracted several times with hot alcohol and the residue is discarded. The combined alcoholic extracts are concentrated *in vacuo* at 60° C. to the consistency of a thick syrup. The thick syrup is extracted repeatedly with water and the residue is discarded. The combined aqueous extracts are concentrated *in vacuo* at 30° C. to a consistency of a thick syrup. The syrup is extracted several times with hot alcohol and the residue is discarded. The combined alcoholic extractions are seeded and set aside for the crystallization of the glycoside. The crystals which adhere to the sides of the container are separated from the alcohol mother-liquor by filtration. They may be readily recrystallized from hot 75% alcohol.

From 45 Kg. of authenticated *Viburnum prunifolium* root bark we obtained 100 Gm. of the crystalline glycoside.

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Correlation of Chemical and Pharmacologic Studies.—Salicin and its aglycone relax the uterus of the rat *in vitro*. It is quite inert on other systems of the body such as blood pressure, respiration, cytochrome system, and perfused amphibian heart. Figure 1 shows the activity of salicin on the human uterus *in vitro*.

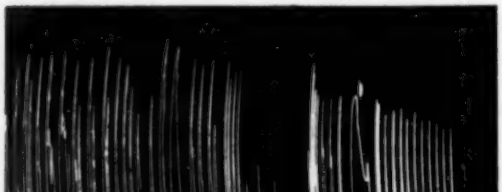

FIG. 1.—Action of Salicin on Human Uterus *in Vitro*.

12:11	1 cc. Salicin, 1%.
12:30	Wash
12:45	0.3 cc Salicin Aglycone, 1%.
1:00	Wash.
1:35	0.5 cc. Salicin Aglycone, 1%.
2:10	Wash.

DISCUSSION

Heyl and Barkenbus (3) made an exhaustive study of this drug and predicted the presence of a glycoside. The glycoside has been isolated and shown to be salicin. This accounts, in a measure at least, for the controversial literature with regard to the efficacy of this drug in the treatment of dysmenorrhea. When given in large quantities it is possible for it to exert an effect, but at best, until proved otherwise, it must be considered a diluted salicin.

Payne (4) and Munch and Youngken (5) have shown viburnum preparations to exhibit a depressor response in the dog upon intravenous injection. The glycoside, salicin, does not share this property even in 5% solution, 0.5 cc. per Kg. in the dog, rabbit, cat, and Macacus rhesus monkey.

A lead subacetate filtrate of the fluidextract (lead removed by hydrogen sulfide and the latter removed by vacuum) gave a depressor response in the dog (6 animals). Upon complete atropinization, 50 to 90% of the depressor response is obliterated in the dog indicating choline or a choline derivative. We are inclined to the view that this depressor response of relatively impure viburnum preparations is due to nonspecific depressor amines.

CONCLUSIONS

1. The glycosidal agent isolated previously from *Viburnum prunifolium* N. F. (1) has been isolated in crystalline form.

2. The polemic question of the therapeutic value of viburnum has been discussed in the light of this finding.

3. In the treatment of dysmenorrhea, we hold the view that preparations of *Viburnum prunifolium* must be looked upon as a dilution of the glycoside, salicin, unless evidence to the contrary is forthcoming.

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Book Review

Pencillin and Other Antibiotic Agents, by WALLACE E. HERRELL. W. B. Saunders Company, Philadelphia, 1945. xv + 348 pp. 15 x 23 cm. Price, $5.00.

Antibiotic agents have been known since the time of Pasteur, but as Dr. Herrell points out so ably, the real science of antibiosis dates back to an accidental contamination of a bacterial culture plate by Fleming in 1928. Reported in 1929, this finding gradually became the stimulus for a long chain of events culminating in the little miraculous packages of penicillin now found in the refrigerators of all pharmacies.

D. Herrell traces the historical development of penicillin from the time when it was literally "grown in bedpans and purified by passing through the Oxford police force" to the present.

The story of the development of penicillin is followed by a chapter on methods of preparing it. Information on the physical and chemical properties of this drug is very difficult to obtain. The chapter dealing with these properties is the most comprehensive the reviewer has seen to date.

The specificity of penicillin *in vitro* and the antibacterial properties *in vivo* are given considerable space. The pharmacologist will be interested in the chapter devoted to absorption, excretion, and distribution. After sections on standardization and dosage follow several chapters on the detailed clinical usage for specific infectious diseases.

The last few chapters are devoted to other antibiotics some of which promise to be of considerable importance when once available in quantity. This book is probably the most authoritative and complete treatise on penicillin now available.—MELVIN W. GREEN.

The Spectrophotometric Assay of Hexestrol and Some of Its Pharmaceutical Preparations*

By Hartmann Goetze and L. D. Seif

The increasing use of hexestrol in medicine necessitates a simple and sufficiently accurate method of analysis for it and its preparations to meet required standards. Much of the standardization is now performed by biological assay (1), by the gravimetric diacetate method (2) or by a colorimetric procedure using a modified Folin–Dennis reagent (3). Biological assays are well known to be lengthy and expensive. The gravimetric method is not very satisfactory for small amounts such as would be found in 0.2-mg. tablets. The color developed with a modified Folin–Dennis reagent is not sufficiently stable to measure accurately with a sensitive photoelectric instrument such as the Beckman spectrophotometer.

EXPERIMENTAL

The method used in our work is carried out on the Beckman spectrophotometer (Model DU) with ultraviolet attachment and is similar to that employed by Elvidge (4). The Beckman is a photoelectric instrument while Elvidge employed a photographic spectrophotometer.

The absorption peaks of hexestrol have previously been reported by Elvidge (4) at 229 mμ and 281 mμ in the ultraviolet range of the spectrum. The absorption curve for hexestrol as determined in this laboratory appears in Fig. 1. The peak used in our work was 279 mμ. The slight shift is probably due to the difference in solvent, the instrument and technique used. Denatured ethanol containing 5% methyl alcohol was employed instead of spectroscopically pure anhydrous ethanol. The individual investigator should determine the suitability of a particular solvent since many denaturants absorb too strongly for practical work with the Beckman instrument in the ultraviolet region.

Two samples of hexestrol prepared in our laboratories and recrystallized six times were used as standards. Neither sample contained more than 0.2% moisture and the melting point of each was 187° C. Five milligrams per 100 cc. of denatured ethanol gave a density reading of 0.632 for each at a peak of 279 mμ.

Twenty-four different batches of hexestrol made in our laboratories were tested by this method. They were precipitated and recrystallized once. The moisture content was less than 1.0% in all samples and the melting points ranged from 184° to 188° C. The results of the spectrophotometric analyses varied from 97.7% to 101.0% of the theoretical when based on the two purified standards.

The method employed in the analysis of compressed tablets was the same as that employed by Elvidge (4) except that denatured ethanol was used. Twenty different batches of 1.0-mg. tablets and twenty-three separate batches of 3.0-mg. tablets varied no more than ±6.0% of the labeled amount.

Elvidge (4) describes a procedure for the analysis of diethylstilbestrol in oil in which the diethylstilbestrol is shaken out of an ether solution with aqueous alkali, made acid and extracted with ether. The ether layer is washed with a bicarbonate solution and the ether solution is evaporated to dryness. The residue is dissolved in spectroscopically pure dehydrated ethanol and the diethylstilbestrol determined on the spectrophotometer. This is applicable to hexestrol but previous experience in analysis of oil solutions of synthetic phenolic estrogens involving this extraction process indicates that quantitative results are not obtained. Consequently, the following procedure was adopted in our work to eliminate unnecessary extractions and washings which interfere with the accuracy of the results.

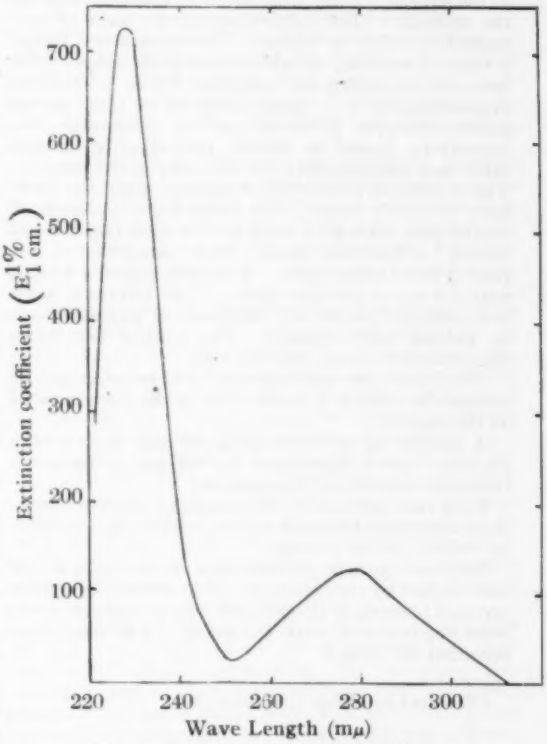

Fig. 1.—Hexestrol Absorption Curve

Analytical Procedure.—Extractions of standard, sample and blank are made simultaneously, and the density determined on the spectrophotometer immediately after extraction and dilution.

* Received March 28, 1945, from the Analytical and Control Department, Research Laboratories, The Wm. S. Merrell Company, Cincinnati, Ohio.

TABLE I.—RESULTS OF SPECTROPHOTOMETRIC ANALYSES OF SOLUTIONS OF HEXESTROL IN OIL

Cc. Sample	5.0 Mg. Standard Density	Sample Density	Labeled Concentration	Mg. Found in Sample	% Hexestrol, Wt./Vol.
5.0	0.597	0.628	1.0 mg./1 cc.	5.25	105
5.0	0.597	0.590	1.0 mg./1 cc.	4.95	99
5.0	0.580	0.574	1.0 mg./1 cc.	4.95	99
5.0	0.580	0.582	1.0 mg./1 cc.	5.01	100.5
5.0	0.600	0.590	1.0 mg./1 cc.	4.92	98.5
5.0	0.600	0.628	1.0 mg./1 cc.	5.23	104.5
5.0	0.570	0.547	1.0 mg./1 cc.	4.81	96
5.0	0.600	0.598	1.0 mg./1 cc.	4.98	99.5
5.0	0.612	0.642	1.0 mg./1 cc.	5.22	104.5
5.0	0.552	0.562	1.0 mg./1 cc.	5.09	102
5.0	0.571	0.610	1.0 mg./1 cc.	5.34	107
1.0	0.580	0.610	5.0 mg./1 cc.	5.25	105
1.0	0.580	0.600	5.0 mg./1 cc.	5.17	103
1.0	0.599	0.618	5.0 mg./1 cc.	5.16	103
1.0	0.490	0.501	5.0 mg./1 cc.	5.13	102
1.0	0.571	0.553	5.0 mg./1 cc.	4.85	97

A standard must be run each time along with the sample and blank. Ten cubic centimeters of peroxide-free ether containing 5.0 mg. of standard hexestrol powder is transferred to a 250-cc. separatory funnel containing the same amount of ampul oil as the sample (of the same batch as used in preparing the ampuls). Ten cubic centimeters more of peroxide-free ether is added. The separatory funnel is rotated until the oil is dissolved in the ether. The hexestrol is shaken out with four 5.0-cc. portions of approximately 1 *N* alkali solution (4 Gm. NaOH pellets dissolved in 100 cc. of 50% ethanol). The separatory funnel is shaken immediately, moderately and continuously for not over eight seconds. The mixture is permitted to separate until the lower layer is nearly clear. The lower layer is drawn off and filtered through a small wetted filter paper into a 100-cc. volumetric flask. This extraction is repeated three more times. The filter paper is washed with 5.0 cc. of distilled water. The filtrate is made acid with 10.0 cc. of 10% HCl, mixed well and made to volume with ethanol. The optical density is determined in 1-cm. quartz cells.

The blank (the same amount and lot of ampul oil without hexestrol) is shaken out in the same manner as the standard.

A sample of oil containing 5.0 mg. hexestrol is pipetted into a separatory funnel and extracted in the same manner as the standard.

Four standards were run simultaneously to determine the consistency of results. They agreed within ±1.0% of the average.

Sixteen separate preparations of hexestrol in oil[1] were tested by this method. The amount of chlorobutanol present in the oil solutions as a preservative does not interfere with the assay. The results are recorded in Table I.

[1] Produced by Loeser Laboratory, Inc.

This method of determination with suitable modification is applicable to pure hexestrol as well as other dosage forms such as tablets. It is also possible to use this spectrophotometric method for diethylstilbestrol and related synthetic estrogens.

SUMMARY

The spectrophotometric analysis of hexestrol and the preparations discussed has proved very satisfactory over a period of time in our Analytical Laboratories. The data, compiled by several technicians, testify to the simplicity and accuracy of the method.

The powder and tablets require about thirty minutes for analysis and the oil solutions about one hour.

1. The practical application of the spectrophotometric method of Elvidge (4) for routine quantitative analysis of hexestrol and hexestrol tablets has been substantially confirmed.

2. A satisfactory method for the analysis of solutions of hexestrol in oil is described.

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The White Rat in the Biological Assay of Senna and Its Preparations*

By Hugh C. Vincent,† C. H. Waldon‡ and C. O. Lee§

Incidental to their study upon precipitation in fluidextract of senna, Kaufman and Lee (1) did some preliminary work on the use of white rats in the bioassay of senna, which was not included in the published report. Wiebelhaus and Lee (2) performed further experiments on the rat assay method. The present paper is based on these previous reports, and presents the results of an effort to work out a suitable bioassay method for senna and its preparations, using the albino rat as the test animal.

EXPERIMENTAL

Healthy, virgin albino rats of the Wistar strain, Denver variety, were used in this study. The sexes were divided as equally as possible in each experiment. Purina Dog Chow Checkers, in either the granular or the cube form, constituted the standard diet during the tests. All animals had been given this diet for at least two weeks prior to their use in the study. Water was supplied freely at all times. The animals were handled enough so that they were tame and were not nervous or excitable during preparations for the tests.

The rats were kept in individual cages made of one-half inch wire screen. A metal pan, which could be lined with paper, was under the screen bottom. The temperature was thermostatically controlled at about 25° C. during the cooler months, when steam heat was used. During this period, the temperature varied appreciably only when a strong wind created drafts in the room. The building was not air conditioned, so that temperatures fluctuated considerably during the hot summer months. Humidity was not controlled.

Alexandria Senna, Lot No. 67627 of Eli Lilly and Co., was used in the project, both as the reference standard and as the crude drug from which the preparations to be tested were made. To prepare the doses, the powdered senna or the preparations were thoroughly incorporated by trituration with the powdered food in a definite ratio, usually 1:100 or 1:200. This stock mixture was prepared to simplify the weighing of the individual doses. The dose of the prepared stock mixture was calculated for each rat on the basis of body weight in kilograms, and weighed on a prescription scale. Sufficient powdered food was added so that there were roughly 5–7 Gm. of it in each dose mixture.

The rats to be used in each test were weighed to the closest 5 Gm., and were then starved for about twenty-four hours. The dose mixtures were then fed to the rats in McCollum feeders in the late afternoon or evening, at which time the doses were most readily eaten. Only about 1% of 3366 individual feedings in the whole project were not eaten.

While the standard diet was being fed to the rats, the feces were quite uniform in appearance. They were firm, and did not adhere to the paper toweling placed below the screen bottoms of the cages. However, there were cases of diarrhea at the beginning of most experiments, and the rats so affected were always eliminated. The incidence of diarrhea was noticeably higher whenever there was a sudden temperature change in either direction, but especially so when it became cooler.

The droppings became softer and adhered to the paper as the main result of feeding senna to the rats. The results of the tests were observed the morning after senna had been administered, and were classed as either positive or negative, with fecal consistency and adherence to the paper as the chief criteria. In order to make the determinations as objective as possible, the doses used were not listed on the charts until after the readings had been made. Any tendency to judge results on the basis of dosage was thus eliminated. The tests were run every seven days.

Size of the Rats.—Female rats weighing 165–230 Gm. and males weighing 175–300 Gm. were used in the tests. Preliminary experiments indicated that animals weighing below these minimum figures were less sensitive to senna, and that animals weighing more than the maximum were considerably more sensitive to senna.

Determination of Minimum Laxative Dose.—The doses administered, in milligrams of senna (or in milligrams of senna represented by doses of the preparations), and the response, in percentage of animals showing positive laxation, were interpreted by standard statistical procedure. The calculated or interpolated dosage which was required to produce laxation in 50% of the animals is called the M. La. D_{50}. The M. La. D_{50} of the reference standard senna was found to be 85.7 mg. per Kg. of body weight. This figure was reasonably constant in most tests, the results of which are grouped together in Table I. The figure was higher in a few tests which are reported separately. The following preparations are also listed in Table I.

Fluidextract of Senna, U. S. P. XII.—Fluidextract of senna was prepared by the official method, and was tested eleven days later. In preparing the dosage mixtures, the fluidextract was first triturated thoroughly with a small portion of the food. The mixture was exposed to the air for two or three hours to permit most of the alcohol to evaporate, before making it up to weight. The M. La. D_{50} of the fluidextract was estimated to be 0.81 cc., representing 810 mg. of senna; it was 10.6% as active as the reference standard.

Autoclaved Senna.—Heat treatment of senna was recommended by Wiebelhaus and Lee (2) as a means of lessening precipitation in the fluidextract. Two

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samples of powdered senna were autoclaved for thirty minutes at 20 lb. pressure (121–127° C.), and assayed after drying. The sample which had been heated after being dampened with an equal weight of distilled water was 17.1% as active as the reference standard. The sample which had been autoclaved dry was 11.4% as active as the standard.

A Senna Unknown.—To aid in testing the accuracy of the bioassay method, one of the authors prepared a mixture of powdered senna, 25%, and food, to be assayed by one of the other investigators. The assay showed that the mixture was 27.2% as active as the standard.

inadequate controls, for which reason the results are not reported in detail. There were indications that each infusion possessed about 60% of its theoretical activity. Alcoholic precipitation of the mucilage did not seem to lessen activity of the infusions. Treatment of the 5% infusion with 0.5% emulsin for twenty-four hours decreased its activity by about 50%, as might be expected from the hydrolyzing action of this enzyme on the senna glycosides (3, 4). Similar treatment with pancreatin, pepsin, papain or malt diastase had no effect.

Bioassay of Isolation Products of Senna.—Straub and Gebhardt (5) stated that the active constituents

TABLE I.—LAXATION IN RATS PRODUCED BY SENNA AND CERTAIN SENNA PREPARATIONS

Test Material	Dose,[a] Mg./Kg.	No. of Rats	Per cent Positive	Calculated M. La. D_{50}, Mg./Kg.	Percentage of Reference Standard Activity
Reference Standard, Alexandria Senna	50	81	29.6	85.7	100
	75	102	30.4		
	100	174	70.0		
	125	293	79.5		
	150	259	86.5		
Fluidextract of Senna[b]	600	14	21.4	810	10.6
	750	16	43.8		
	900	16	56.3		
	1050	8	87.5		
Senna autoclaved damp, 30 min.	400	8	12.5	500	17.1
	500	8	75.0		
	600	8	62.5		
Senna autoclaved dry, 30 min.	400	9	11.1	750	11.4
	500	9	22.2		
	600	9	33.3		
Senna, 25%, plus the food, 75%	250	13	15.4	315	27.2
	300	14	57.1		
	330	10	60.0		
	400	14	85.7		

[a] Doses of preparations refer to quantity of senna represented therein. Each dose was mixed with 5–7 Gm. of the powdered food.
[b] Alcohol was removed by evaporation in air.

Toleration or Sensitization of Rats to Senna.—Sixty rats were divided into four groups as nearly as possible uniform in average weight, in distribution of weight, and in sex. Four doses of senna were administered, the same dose to each group for five tests which were one week apart. The results, given in Table II, show that the M. La. D_{50} varied but little from the 85.7 mg./Kg. previously determined. This would seem to indicate that neither toleration nor sensitization occurred.

TABLE II.—TOLERATION OR SENSITIZATION OF RATS TO SENNA

Test No.[b]	Percentage of Rats Showing Positive Response to Doses of:[a] 50 Mg.	75 Mg.	100 Mg.	125 Mg.	Calculated M. La. D_{50}, Mg./Kg.
I	30.8	21.4	84.6	78.6	84.2
II	25.0	33.3	78.6	85.7	82.8
III	21.4	34.6	90.0	65.4	83.7
IV	26.7	20.0	60.7	92.3	87.6
V	30.8	17.9	75.0	88.5	84.2

[a] Doses are mg. of Alexandria Senna per Kg. body weight; each dose was mixed with 5–7 Gm. powdered food.
[b] Tests were run at 1-wk. intervals, on the same groups of 15 rats.

Aqueous Preparations of Senna.—Freshly prepared 5 and 10% infusions of senna were tested with

of senna are free emodin, a stable anthranol glycoside, and a labile anthranol glycoside, the latter of which was not isolated in pure form. Straub and Triendl (6) reported that the free emodin and the labile glycoside caused undesirable pharmacological effects, and that the stable glycoside was the only effective constituent of senna leaves. F. Hoffman-LaRoche and Co. (7) patented a simplified process for making a relatively pure product containing the stable glycoside. This process was used in making the following products tested in this experiment. One hundred grams of powdered senna were exhausted in a Soxhlet extractor successively with ether, ethanol, and methanol. The powdered extracts, dried *in vacuo*, and the dried marc were tested in a series of 7 assays, the results of which are summarized below in comparison with the results of the similar work by Straub and Gebhardt (5), who used the white mouse as a test animal. The figures represent the activity of the extracts in terms of their percentage of the total activity of senna.

	This Work	Straub and Gebhardt
Ether extract	Negligible	...
Ethanol extract	About 15%	15%
Methanol extract	About 45%	50–60%
Marc	About 15%	20%

The Influence of the Sex of the Rats on Their Response to Senna.—Throughout the course of the project, a record was kept of the varying responses of male and female rats in an attempt to ascertain if the sexes reacted to senna in different degrees. Such a comparison could be made only in those tests in which there was a uniform ratio of the sexes in all groups. Thirty-two tests met that requirement, including 2484 individual feedings (1275 females, 1209 males). Males were more sensitive to senna or its products in 16 of these tests, females in 16. The average variation was 3.98%. It seems safe to conclude that male and female rats, in the weight range used in this work, show an equal cathartic response to senna.

SUMMARY

1. A bioassay procedure for senna and its preparations is outlined, using the white rat as the test animal. The sex of the animals was shown to be a minor factor in their response to senna. The rats did not develop toleration or sensitization to senna in 5 consecutive weekly tests.

2. Several preparations of senna, including the fluidextract, 5% and 10% infusions, and a diluted sample of powdered senna, were tested.

3. The rat method was used to determine the laxative value of the methanol, ethanol, and ether extracts described by Straub and co-workers. The results were in reasonable agreement with their findings.

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Spirit of Chloroform, N. F. VII*

By Samuel W. Goldstein

A number of commercial samples of Spirit of Chlorofom were tested and most of them failed to meet the minimum official requirement for chloroform as given in the National Formulary VII. In order to determine whether the recognized standard is too stringent and whether the assay procedure is sufficiently accurate, the following study was made.

Spirit of Chloroform U. S. P. XI was assayed by heating for four hours a mixture of the sample and alcoholic potassium hydroxide (3 in 10) in a flask connected with a reflux condenser. The resulting potassium chloride was then determined, after dilution, by the volumetric Volhard procedure. Chloroform Liniment U. S. P. XI was subjected to a preliminary dilution and distillation; the distillate then being treated as in the assay for Spirit of Chloroform.

Publications by Kunke (1), Beal and Szalkowski (2) and Goldstein and Reindollar (3) showed that a number of the steps in the official procedure for Chloroform Liniment were unnecessary, and that substitution of a pressure bottle for the flask and reflux condenser shortened the time required for the assay by more than three hours and also yielded more accurate results. The U. S. P. XII Subcommittee also studied a simple direct distillation procedure for the assay of Chloroform Liniment which requires only the dilution of 20 cc. of the sample with 10 cc. of alcohol and 20 cc. of water, and distillation using a dephlegmator (4). The distillate is collected in water in a cooled carbon tube in which, after agitation, the volume of chloroform is read directly. The latter procedure was made official in the U. S. P. XII.

Spirit of Chloroform was dropped from the United States Pharmacopœia in the twelfth revision and therefore the committee did not consider altering its assay. The preparation was included in the seventh edition of the National Formulary and the

* Received Feb. 6, 1945, from the laboratories of the Bureau of Chemistry of the State of Maryland Department of Health, Baltimore.

assay procedure given in the U. S. P. XI was retained.

The shorter procedure published by the present author for chloroform Liniment (3) is applicable to Spirit of Chloroform (2). The belief that the U. S. P. XII assay for Chloroform Liniment could be applied to the Spirit, and the possibility that the modified Randall procedure (5) for the assay of volatile oils in spirits, which we have found to work well also with Spirit of Camphor (6), might be used for Spirit of Chloroform led to the following experiments.

EXPERIMENTAL

Spirit of Chloroform. Sample A.—22.3098 Gm. of chloroform (C. P.) were weighed and made up to 250 cc. with alcohol at 25° C. This is equivalent to 8.9239 Gm. or 6.038 cc. of chloroform per 100 cc. of spirit.

Sample B.—22.2328 Gm. of chloroform (C. P.) were weighed and made up to 250 cc. with alcohol at 25° C. This is equivalent to 8.8931 Gm. or 6.017 cc. of chloroform per 100 cc. of spirit.

Sample C.—22.2941 Gm. of chloroform (C. P.) were weighed and made up to 250 cc. with alcohol at 25° C. This is equivalent to 8.9176 Gm. or 6.034 cc. of chloroform per 100 cc. of spirit.

Other samples were purchased at different drug-stores.

Method I. Modified Volhard Procedure.—Transfer 5 cc. of Spirit of Chloroform to a pressure (or citrate) bottle containing 50 cc. of an alcohol solution of potassium hydroxide (3 in 10), holding the end of the pipette just above the alkali solution. Seal the bottle and place in a water bath, heat the water to boiling, and maintain at the boiling temperature for a half hour. Proceed as directed in the assay under Spirit of Chloroform N. F. VII, page 385, beginning with the words "Cool the mixture," in the fifth line of the assay. The 100-cc. aliquot was transferred directly to a 250-cc. volumetric flask without heating to expel the alcohol (1), and nitric acid was used in place of diluted nitric acid.

Method II. Distillation Procedure.—The method is the same as that given under Chloroform Determination in the U. S. P. XII, page 564, with the following changes: Use a 200-cc. extraction or Erlenmeyer flask. Place 50 cc. of distilled water in the flask and add 50 cc. of Spirit of Chloroform, holding the end of the pipette just below the surface of the water. The distillate is collected in a calibrated carbon tube and the volume red at 25° C.

Method III. Modified Randall Procedure.—The procedure given in the assay for Spirit of Peppermint U. S. P. XII, page 431, was used with the following changes: Add acidified calcium chloride solution almost to the shoulder of the Babcock bottle, then add the kerosene and 8 cc. of Spirit of Chloroform. Stopper with a cork wet with calcium chloride solution, agitate vigorously for one minute, then add sufficient of the calcium chloride solution to bring the liquid to the top of the calibrated portion of the neck of the bottle. After centrifugation, bring the temperature to 25° C., then read the volume of oil. The number of divisions corresponding to the chloroform present was multiplied by the factor 3.87. This factor was obtained by dividing the average number of Babcock divisions, corresponding to the chloroform in the samples of the control spirit, into the weight of chloroform in 100 cc. of Spirit of Chloroform.

Where it was desirable to express the grams of chloroform as cubic centimeters of chloroform, or vice versa, 1.478 was used as the specific gravity of chloroform.

TABLE I.—DETERMINATION OF CHLOROFORM IN CONTROL SPIRITS OF CHLOROFORM

Sample	Method	Cc. $CHCl_3$/100 Cc.	Gm. $CHCl_3$/100 Cc.	% Recovery
A	I	5.76	8.509	95.35
A	I	5.72	8.457	94.76
A	I	5.78	8.541	95.71
A	I	5.78	8.541	95.71
A	I	5.76	8.509	95.35
A	I	5.76	8.509	95.35
A	II	6.00	8.868	99.37
A	II	5.93	8.765	98.21
A	III	5.94	8.785	98.44
A	III	5.97	8.824	98.88
A	III	6.05	8.940	100.18
A	III	6.05	8.940	100.18
A	III	6.02	8.901	99.74
A	III	6.05	8.940	100.18
B	II	5.95	8.794	98.89
B	II	5.94	8.779	98.72
B	II	5.90	8.720	98.06
B	II	5.93	8.765	98.55
C	I	5.79	8.562	96.01
C	I	5.76	8.509	95.42
C	I	5.76	8.509	95.42
C	I	5.76	8.509	95.42
C	II	6.00	8.868	99.44
C	II	6.00	8.868	99.44
C	II	5.86	8.661	97.12
C	II	6.00	8.868	99.44
C	III	6.07	8.978	100.68
C	III	6.00	8.862	99.38
C	III	5.94	8.785	98.51
C	III	5.97	8.824	98.95

DISCUSSION

The average per cent recoveries are: Method I, 95.45 per cent; Method II, 98.72 per cent; Method III, 99.51 per cent. It would appear that the modified Randall procedure gives results very close to the true chloroform content of Spirit of Chloroform, and that it would therefore be the favored method. However, Method II gives very good results and utilizes a procedure already recognized by the U. S. P. XII for the determination of chloroform. Furthermore, its utter simplicity and convenience of manipulation once the apparatus is assembled, and its economy of time and reagents make it the method of choice.

Beal and Szalkowski (2) working with control samples reported 95.84 per cent and 98.32 per cent recoveries of chloroform from

TABLE II.—DETERMINATION OF CHLOROFORM IN COMMERCIAL SAMPLES OF SPIRIT OF CHLOROFORM

Sample	Method	Cc. $CHCl_3$/-100 Cc.	Gm. $CHCl_3$/-100 Cc.	Variation of Recovered Amount from Prescribed Amount, %
11727 C	I	5.67	8.378	− 5.58
11727 C	II	5.64	8.336	− 6.00
11733 C	I	5.65	8.353	− 5.83
11733 C	II	5.60	8.277	− 6.67
11737 C	I	5.81	8.587	− 3.18
11737 C	II	5.86	8.661	− 2.33
11743 C	I	5.38	7.945	−10.33
11743 C	II	5.40	7.981	−10.00
11749 C	I	5.18	7.662	−13.67
11749 C	II	5.24	7.745	−12.67
11776 C	I	5.54	8.192	− 7.67
11776 C	II	5.74	8.484	− 4.33
11776 C	III	5.89	8.708	− 1.83
11787 C	I	5.70	8.428	− 5.00
11787 C	II	5.78	8.543	− 3.67
11787 C	III	5.87	8.669	− 2.17
11792 C	I	6.05	8.935	+ 0.83
11792 C	II	6.22	9.193	+ 3.67
11792 C	III	6.28	9.288	+ 4.67
11795 C	I	6.05	8.945	+ 0.83
11795 C	II	6.04	8.927	+ 0.67
11795 C	III	6.21	9.172	+ 3.50
11800 C	I	5.49	8.119	− 8.50
11800 C	II	5.60	8.277	− 6.67
11800 C	III	5.79	8.553	− 3.50

the spirit, using the present official procedure; and 96.39 per cent and 98.45 per cent recoveries using the pressure bottle modification. They also reported values of 8.442 Gm. per 100 cc. and 8.362 Gm. per 100 cc. for two Spirits of Chloroform prepared by two members of the U. S. P. Revision Committee. These preparations were assayed by the official procedure and the results obtained indicated that neither spirit met the present official minimum requirement of 8.5 Gm. of chloroform per 100 cc. of spirit, and that they were, respectively, 4.80 per cent and 5.70 per cent below the prescribed amount of chloroform. The present official tolerance allows deviations of −4.15 per cent and +4.31 per cent from the prescribed amount of chloroform, and the results in Table II show that by the modified official assay procedure three out of ten commercial samples met the required minimum and none reached the allowed maximum. Four preparations met the minimum requirement by Method II. We are of the opinion that the official tolerance for this preparation is too stringent. A deviation from the mean of ±10 per cent is allowed in most of the spirits of essential oils, and deviations of −8.0 per cent and +4.0 per cent from the prescribed amount are allowed in Spirit of Camphor. A tolerance of 10 per cent below the prescribed amount of chloroform is allowed in Chloroform Liniment, while only 1.67 per cent is allowed above the prescribed amount. A tolerance of 7.5 per cent below the prescribed amount and 5.0 per cent above the prescribed amount of chloroform in Spirit of Chloroform would be fairer than the present official tolerance for this preparation. It is recommended that the chloroform content be expressed as cubic centimeters instead of grams. The rubric would then read as follows: "Spirit of Chloroform contains, in each 100 cc., not less than 5.55 cc. and not more than 6.30 cc. of $CHCl_3$ at 25° C." The volume of chloroform obtained by the distillation procedure would then be multiplied by 2 and reported directly.

SUMMARY

1. A number of samples of Spirit of Chlorofom N. F. VII have been assayed by three different procedures.

2. Substitution of the U. S. P. XII procedure, with slight modification, for the determination of chloroform in place of the assay official in the N. F. VII is recommended.

3. The limits of tolerance have been found to be too stringent for Spirit of Chloroform N. F. VII, and the following rubric is recommended for the official monograph: "Spirit of Chloroform contains, in each 100 cc., not less than 5.55 cc. and not more than 6.30 cc. of $CHCl_3$ at 25° C."

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A Chemical Study of Aromatic Spirit of Ammonia*

Charles F. Poe and Martin E. Hultquist

Aromatic Spirit of Ammonia first appeared as an official preparation in the London Pharmacopœia of 1721 under the name "Spiritus Salis Volatilis Oleosis." The present name was first adopted in the London Pharmacopœia in 1809.

The percentage of ammonia in the spirit is subject to considerable variation because of the use of inferior grades of ammonium carbonate in the preparation of the spirit, the loss of ammonia on standing, or adulteration by the manufacturer. In addition, components other than ammonia might be present in amounts below those required in the official preparation.

The investigation presented in this publication was undertaken to study the methods of analysis used in the detection of adulteration of the spirit, and to study the changes in composition of the spirit with age.

EXPERIMENTAL

Methods and Procedures.—A number of samples of ammonium carbonate were obtained from different sources and these were used in the preparation of different lots of aromatic spirit of ammonia.

The samples were analyzed immediately for total ammonia, volatile oil, and alcohol content; and the specific gravity and refractive index were determined. Each sample was divided into four portions; two of these were placed in glass-stoppered containers and two were placed in cork-stoppered bottles. One-half of each set of samples was placed in a dark room, the temperature of which was maintained at 20° C. The other samples were kept on a shelf in the laboratory out of direct sunlight. At stated intervals the ammonia content of each sample was redetermined.

Total ammonia was determined by three methods:

(*A*) A given amount of spirit was measured with a pipette into a glass-stoppered Erlenmeyer flask containing an excess of standard H_2SO_4. The titration was completed with standard NaOH, methyl orange being used as an indicator.

(*B*) A given amount of spirit was subjected to steam distillation, and the ammonia was collected in standard acid solution. The excess acid was neutralized as in (*A*).

(*C*) Another sample was made acid with H_2SO_4, the volatile oils were distilled with steam, then an excess of NaOH was added and the ammonia separated by steam distillation as in (*B*).

The alcohol was determined as follows:

(*A*) By the method recommended by the U. S. Pharmacopœia X.

(*B*) A given sample was made acid with H_2SO_4, 2 Gm. of kaolin was added and the mixture diluted to a given volume. After shaking, the mixture was filtered and the alcohol in an aliquot part was determined.

(*C*) The method of Booth (1) was used. In this method the volatile oil was not removed before distillation.

The volatile oil was determined by the Mitchell method (2).

The specific gravity and refractive index were determined at 25° C.

TABLE I.—COMPOSITION OF AROMATIC SPIRIT OF AMMONIA

Sample No.	Sp. Gr.	Ref. Index	Ammonia Gm. 100 Cc. Method			Alcohol in % by Vol. Method			Total Oils,
			A	*B*	*C*	*A*	*B*	*C*	%
1	0.8964	1.3662	1.90	1.95	1.91	64.70	66.54	...	1.22
2	0.8961	1.3688	1.86	1.84	1.88	64.74	66.28	...	1.20
3	0.8946	1.3667	1.91	1.88	1.90	65.75	66.56	66.60	1.21
4	0.8948	1.3667	1.91	1.87	1.89	65.82	66.72	66.86	1.20
5	0.8949	1.3664	1.95	1.93	1.94	65.88	66.70	66.81	1.20
6	0.8952	1.3667	1.95	1.93	1.94	65.48	66.72	66.70	1.19
7	0.8949	1.3665	1.84	1.82	1.84	65.90	66.72	66.70	1.20
8	0.8953	1.3668	1.94	1.93	1.93	66.05	66.60	66.80	1.20
9	0.8954	1.3664	1.96	1.94	1.96	66.12	66.48	66.87	1.22
10	0.8951	1.3666	1.87	1.87	1.87	66.05	66.60	66.90	1.19
11	0.8956	1.3667	1.90	1.90	1.91	65.89	66.28	66.73	1.20
Av.	0.8953	1.3668	1.91	1.90	1.91	65.67	66.56	66.77	1.20

RESULTS

The results of the analyses of eleven samples of spirit are presented in Table I. Each sample was made with a different lot of ammonium carbonate.

By using U. S. P. chemicals and considering the limits of purity permitted, the amount of ammonia should be between 1.84 and 2.00 Gm. per 100 cc. of spirit. There should be 66.50% alcohol and 1.2% volatile oil, by volume.

The three methods used for total ammonia give equally accurate results. The method of direct titration is accurate and saves the time of steam dis-

* Received July 12, 1944, from the Department of Chemistry, College of Pharmacy, University of Colorado, Boulder, Colo.

TABLE II.—ANALYSIS OF SPIRITS MADE WITH VARIOUS STRENGTHS OF ALCOHOL

Sample No.	Sp. Gr.	Ref. Ind.	Alcohols in % by vol. with Method A	B	C	Theory, %
1	0.9395	1.3620	46.97	47.66	47.77	47.50
2	0.9292	1.3635	51.25	52.16	52.30	52.25
3	0.9172	1.3647	56.00	56.80	56.07	57.00
4	0.9047	1.3656	60.50	61.56	61.40	61.75
5	0.8805	1.3671	68.60	71.12	70.86	71.25

TABLE III.—THE AMMONIA CONTENT OF SAMPLES KEPT IN GLASS-STOPPERED BOTTLES

Sample No.	Original	Light 6 Mo.	12 Mo.	24 Mo.	36 Mo.	Dark 6 Mo.	12 Mo.	24 Mo.
1	1.88[a]	1.87	1.80	1.73	1.59	1.87	1.83	1.80
2	1.89	1.87	1.79	1.66	1.33	1.85	1.77	1.72
3	1.82	1.81	1.70	1.69	1.59	1.82	1.77	1.74
4	1.68	1.68	1.67	1.60	1.50	1.68	1.68	1.61
5	1.90	1.84	1.76	1.70	0.63	1.72	1.66	1.63
Av.	1.83	1.81	1.74	1.68	1.33	1.79	1.74	1.70

[a] Grams of ammonia per 100 cc.

TABLE IV.—THE AMMONIA CONTENT OF SAMPLES KEPT IN CORK-STOPPERED BOTTLES

Sample No.	Original	Light 6 Mo.	12 Mo.	24 Mo.	36 Mo.	Dark 6 Mo.	12 Mo.	24 Mo.
1	1.88[a]	1.81	1.75	1.22	1.09	1.84	1.75	1.38
2	1.89	1.82	1.72	1.50	1.34	1.83	1.78	1.60
3	1.82	1.77	1.68	1.12	0.73	1.76	1.69	1.28
4	1.68	1.65	1.59	1.28	1.19	1.65	1.62	1.42
5	1.90	1.79	1.73	1.48	1.24	1.81	1.73	1.40
Av.	1.83	1.77	1.69	1.32	1.12	1.78	1.71	1.42

[a] Grams of ammonia per 100 cc.

TABLE V.—AMMONIA CONTENT OF SAMPLES KEPT IN 300-CC. GLASS-STOPPERED BOTTLES IN THE LIGHT

Sample No.	Original	12 Mo.	24 Mo.	40 Mo.
1	1.80[a]	1.78	1.77	1.60
2	1.81	1.80	1.78	1.60
3	1.89	1.86	1.86	1.56
4	1.89	1.88	1.87	1.80
5	1.94	1.90	1.90	1.73
6	1.87	1.83	1.81	1.62
7	1.82	1.80	1.76	1.67
Av.	1.86	1.84	1.82	1.65

[a] Grams ammonia per 100 cc.

tillation. The U. S. P. method for alcohol gives consistently low results. The method of Booth (1) for alcohol has the disadvantage of having the volatile oils come over with the alcohol. In this method accurate results are obtained only as long as the gravity for the alcohol mixture is the same as the gravity of the volatile oils.

In order to test the accuracy of the method of determining alcohol, a number of spirits were prepared with different percentages of alcohol. The results are given in Table II. In the preparation of the sample with 57% or less alcohol, part of the volatile oils was precipitated. In the sample with 71.25% alcohol, the ammonium carbonate was partly precipitated. As was found with the previous alcohol determinations, the U. S. P. X method gave low results. Method *B* gave very satisfactory results. The results with the Booth Method (*C*) were found to be irregular because of the distillation of the volatile oils with the alcohol.

Various samples were tested for keeping quality after being kept in the light and dark for various lengths of time. The percentages of ammonia only were determined. Table III gives the results for samples kept in glass-stoppered bottles. The samples stored in the light were analyzed after six, twelve, twenty-four, and thirty-six months. The samples stored in a dark room were analyzed after six, twelve, and twenty-four months. From the table it will be noted that during the first year very little ammonia was lost in the samples stored either in the light or dark. Also, the samples kept for twenty-four months showed a small loss of ammonia.

Some of the same samples also were placed in cork-stoppered bottles and stored in the light and dark. The results of these tests are presented in Table IV. The keeping qualities of the spirits in cork-stoppered bottles are satisfactory both in the light and the dark for the first twelve months, but a little less satisfactory than the keeping qualities in glass-stoppered bottles. It was noted that the cork stoppers deteriorated very rapidly and it was necessary to replace them frequently. This operation caused some loss of ammonia.

A separate set of samples was prepared and placed in 300-cc. Erlenmeyer flasks with ground-glass stoppers. The results showing the ammonia content during forty months' storage in the light are given in Table V. The keeping qualities of the samples of the spirit were very good even for forty months.

CONCLUSIONS

1. The methods of analysis of Aromatic Spirit of Ammonia have been studied.
2. The keeping qualities of the spirit stored in glass-stoppered containers in the light and dark have been found to be satisfactory for at least twenty-four months.
3. The keeping qualities of samples stored in cork-stoppered bottles for over twelve months have been found to be satisfactory.

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Hydrogenated Castor Oil as an Ointment Base. VII. Sulfated Product in Antiseptic Ointments*

By George W. Fiero† and Ted A. Loomis

The use of sulfated hydrogenated castor oil as an ointment base was reported in a previous paper (1). Because it is hydrophylic, it would appear that it might have better properties than the official bases for antiseptic medicaments, particularly in cases where the antiseptic is soluble in water.

EXPERIMENTAL

Ointments were prepared using the same strength of active constituent as the official ointments, substituting sulfated hydrogenated castor oil[1] for the official base. In the case of water-soluble medicaments, they were dissolved or moistened with water before incorporating with the base. In addition to the plain sulfated hydrogenated castor oil, ointments were prepared using the cream base suggested in a previous paper (2):

Diethylene glycol monostearate	10 Gm.
Sulfated hydrogenated castor oil	20 Gm.
Petrolatum	30 Gm.
Water	40 Gm.

The agar cup method (3) of the Food and Drug Administration was employed, as described in a previous paper (4). The medium was adjusted to a *p*H of about 6.0 to more nearly simulate that of the skin. The extent of free zone surrounding the ointment is an indication of relative antiseptic activity. The test is relative, the extent of free zone varying with the particular culture of Staphylococcus aureus employed. Data are shown in Table I.

Since the addition of 25% of petrolatum is recommended to reduce the stickiness of the base, bacteriological tests using this base were performed. The free zone was found to be approximately equal to that of plain sulfated hydrogenated castor oil.

TABLE I.—ANTISEPTIC ACTION OF OINTMENTS[a]

Ointment	Official Base	SHCO	"Cream" Base
Ammoniated Mercury	7	14-15	15-16
Boric Acid	0	4	6
Calomel	2.5	3	6
Calomel, Colloidal	4- 6	4- 5	6
Iodine	18	28	...
Phenol	0	0- 2	1- 2
Pine Tar	3- 4	4- 5	7- 8
Red Mercuric Oxide	5- 6	9-11	10
Yellow Mercuric Oxide	4- 5	14-15	8-10
Whitfield	11-12	10-12	15
Whitfield, half-strength	8- 9	7- 9	13
Proprietary A	7- 8	9-10	...
Proprietary B	3- 4	5- 7	9-10

[a] Data expressed in mm. of antiseptic zone surrounding the ointment.

SUMMARY

Sulfated hydrogenated castor oil and an emulsified base consisting of diethylene glycol monostearate 10 per cent, SHCO 20 per cent, petrolatum 30 per cent, and water 40 per cent, were substituted for the official ointment bases in several antiseptic ointments. Bacteriological tests indicate that the antiseptic value of these ointments is equal to or superior to the official ointments.

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† Chief, Pharmaceutical, Insecticide and Cosmetic Chemicals Unit, Office of Civilian Requirements, War Production Board, Washington, D. C.

[1] Manufactured by National Oil Products Co., Harrison, N. J. Known also as hydroxystearin sulfate.

Evaluation of Some Liquid Antiseptics*

By Alvin R. Whitehill

It has been conventional procedure to evaluate antiseptics by testing their germicidal power against Staphylococcus aureus. For the legal definition of an antiseptic, this method is perhaps adequate. However, a large number of these products are sold on the market to disinfect abraded skin, mucous membranes and the eye. Such an antiseptic should be nonirritating to the living tissue, and, at the same time, should kill all organisms which may cause infection.

To the author's knowledge there is no compound which has germicidal powers that will not cause irritation to exposed tissues if applied in a concentrated solution. There are compounds which, when diluted sufficiently with the proper solvent, will cause little irritation and at the same time retain some germicidal powers. Most of the antiseptics sold for application to living tissue have as their active ingredient a compound of this type. Some compounds, when diluted to a nonirritating concentration and still retaining bacteriostatic properties, are efficient only when applied in salves or ointments where they can prevent increase of harmful bacteria over a relatively long period of time. When compounds of this type are used, the amount absorbed by the skin should not cause injury to the host.

The test for irritation used in this investigation is not adequate to cover such cases. This is a comparison of the efficiency of several liquid antiseptics, bought on the open market, which are intended for use on abraded skin, mucous membranes, or in the eye. In order to critically evaluate these products, that antiseptic may be considered best whose lowest nonirritating dilution kills a given amount of S. aureus culture in the least time.

EXPERIMENTAL

Method for Determining Irritation.—No standard method for determining the concentration of a compound which is irritating to living tissue has yet been generally accepted. Lambert (1, 2) used the minimum concentration which stops the growth of human and rabbit cells in tissue culture as a criterion. Buchsbaum (3) and Salle (4) have used the killing of a chick embryo as an index, while Bronfenbrenner, *et al.* (5), and Ely (6) have used the inhibition of liver tissue respiration to determine the toxic end point. Welch and Brewer (7) have proposed still another measure of toxicity, later used by Hirsch and Novak (8) with modification, namely, the inhibition of phagocytic activity.

Each of the above methods for determining injury has its own merits and gives a purely objective measure useful in routine laboratories. The author uses a method which is more in agreement with that which is used at present by the United States Food and Drug Administration (9). The principles of this method are based on the fundamental assumption that irritation is too general a term to be measured by any one criterion, such as phagocytic retention or reduced liver respiration. Equally irritating compounds may show injury by widely divergent means, hence irritation must be judged by the degree of total injury. Calvery (9) has found that best results are obtained by devising numerical systems, the value of which depends on the sagacity with which the worker weighs the different types of injury. Such a method is only semi-objective, but is a valuable compromise between the purely objec-

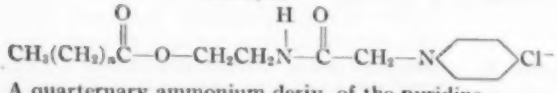
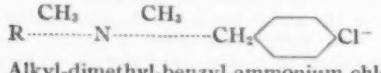
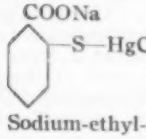
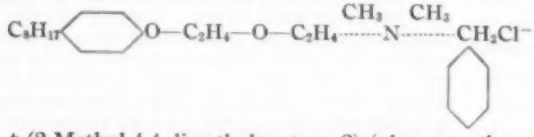
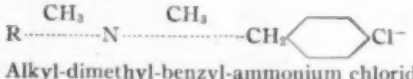
TABLE I.—DETERMINATION OF MAXIMUM NONIRRITATING CONCENTRATION

		Rabbit		Guinea Pig	
Antiseptic[a]	Dilution	1 Hr.	18 Hr.	1 Hr.	18 Hr.
I	1:1000	2+	1+	1+	0
	1:2000	1+	0	1+	0
	1:3000	1+	0	0	0
	1:4000	1+	0	0	0
II	1:1000	3+	1+	3+	1+
	1:2000	2+	1+	2+	0
	1:3000	2+	0	1+	0
	1:4000	1+	0	1+	0
III	1:500	3+	1+	1+	1+
	1:1000	1+	0	1+	0
	1:1500	1+	0	1+	0
	1:2000	1+	0	1+	0
IV	1:1000	2+	1+	1+	1+
	1:1500	2+	0	0	1+
	1:2000	1+	0	1+	0
	1:2500	1+	0	1+	0
V	1:3000	2+	2+	1+	1+
	1:5000	2+	1+	1+	0
	1:8000	1+	1+	1+	0
	1:10,000	1+	0	1+	0
VI	1:1000	1+	1+	1+	1+
	1:2000	1+	0	1+	1+
	1:3000	1+	0	1+	0
	1:4000	1+	0	1+	0
VII	1:100	3+	1+	3+	1+
	1:200	2+	1+	2+	0
	1:300	1+	0	1+	0
	1:400	1+	0	1+	0
VIII	1:8000	3+	3+	3+	4+
	1:10,000	3+	3+	3+	2+
	1:12,000	2+	1+	2+	2+
	1:15,000	1+	0	2+	0
IX	1:800	4+	4+	4+	4+
	1:1000	3+	4+	3+	4+
	1:1200	2+	0	2+	0
	1:1500	1+	0	0	0

[a] The antiseptics listed were purchased on the open market.

* Received Sept. 1, 1944, from the Biology Laboratory of the Illinois Institute of Technology, Chicago, Ill.

TABLE II.—BACTERIAL DISINFECTION AT MAXIMUM NONIRRITATING CONCENTRATION

	Antiseptic	Maximum Nonirritating Concentration of Active Ingredient	Time in Minutes Required to Kill Staph. Aureus at 37° C.
I	 A quarternary ammonium deriv. of the pyridine-betaine type	1:2000	0.5
II	 Alkyl-dimethyl-benzyl-ammonium chloride (Brand name 1)	1:3000	0.5
III	 Sodium-ethyl-mercuri-thiosalicylate	1:1000	60.0
IV	 *p*-(2-Methyl-4,4-dimethylpentano-2) (phenoxy-ethoxy-ethyl) dimethyl benzyl ammonium chloride	1:1500	20.0
V	$C_6H_5HgOH—C_6H_5HgNO_3$ Phenyl Mercuric Nitrate	1:10,000	22.5
VI	Acriflavine	1:2000	12.0
VII	$AgNO_3$ Silver Nitrate	1:300	45.0
VIII	 Alkyl-dimethyl-benzyl-ammonium chloride (Brand name 2)	1:20,000	13.5
IX	$HgCl_2$ Mercuric Chloride	1:1200	5.0

tive and subjective methods, and is used here.

Technique of Determining Lowest Nonirritating Concentration.—By comparable experiments on abraded skin, mucous membranes and the eye, it was found that the eye was the most sensitive indicator and could be tested more easily. Progressive dilutions of the test antiseptics were made in *M*/15 phosphate buffer at a *p*H of 7.4. Each dilution was tested in the conjunctivae of a 2-Kg. albino rabbit and checked on the eyes of a 350-Gm. guinea pig. Two drops were instilled successively at five-minute intervals until six instillations had been administered. Into the other eye of the animal was administered a corresponding dose of *M*/15 phosphate as a control. The test eye was compared to the control eye at one hour and eighteen hours after the last instillation. In rating the degree of injury the following scale was used:

Photophobia	1
Discharge	1
Inflammation	2
Edema	3
Cloudy cornea	4

The total injury was the addition of the separate types of injury. By using this method it was found that one did not have to judge the degree of any one separate component because a severe case of any one criterion was accompanied by one or two of the other symptoms and thus the degree of any one was automatically compensated for in the final score. This scoring system was set up six months after the original work was completed so it was only used to refine the purely subjective impression. For this reason, only limiting concentrations are shown in Table I. No concentration used was high enough

in the refined test to cause clouding of the cornea.

Determination of the Germicidal Power.—The standard method for determining the germicidal power of an antiseptic (10) was not used. It was felt that conditions more closely comparable to those under which the antiseptic reacts in the eye would be a better test medium, since the efficiency of the antiseptics considered in this paper depends upon their killing power in the presence of living tissue. To this end the protein content, *p*H and number of organisms were adjusted to more closely fit natural conditions in the eye.

An actively growing culture of Staphylococci aureus (F. D. A. Strain 209) was suspended in normal saline solution and the suspension centrifuged at a low speed for three minutes to remove larger clumps. The supernatant liquid was then reduced with saline until approximately 7 million organisms were present per ml. of suspension as determined by turbidity measurements. The medium in which the disinfectant tests were performed was designed to resemble tears as closely as practicable. According to Ridley (11) the composition of tears is closely approximated by a mixture of 10 per cent blood serum in physiologic saline. Since *p*H is one of the important factors governing disinfection it was necessary to substitute a *M*/15 phosphate buffer (*p*H 7.4) for saline.

Inactivated horse serum was used as a source of protein. This was diluted with the buffer and added to the chemical under test so that the final concentration when the organisms were added would be the threshold value (irritation in one hour but not in eighteen) of the compounds shown in Table I. This mixture was placed in a 37.5° C. water bath for ten minutes. One cubic centimeter of the suspension of organisms was added and a loopful of the resulting mixture was transferred into sterile nutrient broth at half-minute intervals until the termination of the test.

DISCUSSION OF RESULTS

From the results tabulated in Table II the several commercial antiseptics are rated in order of the killing time of the maximum nonirritating concentration of their active ingredients. Judged by this criterion, the so-called "cationic substances" make the best active ingredients for antiseptics. In the case of two, the killing is practically instantaneous. The one exception is "Roccal," a technical grade of "Zephiran chloride." Its higher toxicity is apparently due to impurities associated with the cationic substance itself. The organic mercury compounds are the poorest listed here; in fact, their killing time is so long they would be of value only in forms of wet packs or when incorporated in ointments.

SUMMARY

1. Several commercial antiseptics, which are sold for the purpose of disinfecting abraded skin, mucous membranes and eyes, were rated according to the time which it required their nonirritating concentration to kill Staphylococcus aureus.

2. It was found by this criterion the cationic substances make the best active ingredients while the organic mercury compounds are the poorest.

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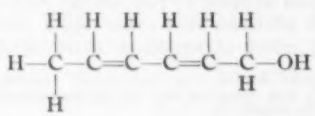
Hexadienol, A Locally Acting Diaphoretic and A New Diagnostic Agent*

By Theodore Koppanyi

Hexadienol, a commercial diene-alcohol (a hexitol) of the general formula $C_nH_{2n-3}OH$, is formed when the hydrocarbon tetracosane is heated together with air in the presence of a catalyst. The reaction is primarily one of oxidation of the long tetracosane chain with subsequent fragmentation into four units, each of six-carbon atoms in a chain ($2C_{24}H_{50} + 9O_2 \xrightarrow[\text{cat.}]{} 8C_6H_{10}O + 10H_2O$).

The following structural formula has been tentatively proposed for hexadienol:



Hexadienol is an amber-colored semi-

* Received Jan. 6, 1945, from the Dept. of Pharmacology and Materia Medica, Georgetown University School of Medicine, Washington, D. C.

solid substance with a terpene-like odor and begins to soften at 72° C. and liquefies at 89° C. Its specific gravity is 0.894; it is not soluble in water, but is miscible with organic solvents and vegetable oils (1).

Applied to the human skin, hexadienol induces localized sweating over the area covered. This observation has already been made in a purely clinical study by Levine (2) who also drew attention to the fact that it does not produce hyperemia and postulated that the sudorific action is due either to stimulation of sympathetic fibers or the sweat glands themselves. Since no previous attempt had been made to study the diaphoretic action of hexadienol, it was decided to undertake a pharmacological analysis of this action and to utilize the results in devising a clinical test for demonstrating local anhydrotic areas in the skin.

EXPERIMENTAL

Hexadienol for local applications was used in its commercial form[1] of which 98% is $C_6H_{10}O$ and the remaining 2% consists of unreacted tetracosane, lauryl alcohol and some esters. For injections commercial hexadienol was dissolved in cottonseed oil; for every 100 Gm. of hexadienol 200 Gm. of cottonseed oil was used, thoroughly mixed and heated over a water bath until the mixture was completely liquefied. This liquefaction occurs at an average temperature of 40° C.

A. The Diaphoretic Action of Hexadienol in Cats and Human Subjects.—In twelve unanesthetized cats hexadienol in doses of 150 mg. and 300 mg. per Kg. of body weight[2] dissolved in cottonseed oil was injected intravenously. These injections produced a temporary depression from which the animal generally recovered in fifteen minutes. There were no nausea, vomiting or convulsions, but in some instances the animals showed rigidity. In every case there was profuse sweating on the foot pads observable with the naked eye, lasting from thirty minutes to two hours.

Hexadienol (total amounts from 0.1 to 0.3 cc. of a 33.3% solution) was also injected into the foot pads of eight cats and in each case large droplets of sweat were seen a few minutes after injection. When the same amounts of physiological salt solution were injected into another foot pad of the same animal, the sweat droplets, occasionally produced, were far smaller and fewer than on the opposite foot pad injected with hexadienol. The comparison of these effects can most conveniently be made under a magnifying glass. Local application with friction of pure commercial hexadienol on the skin of the foot pad produced sweating only in three of twelve cats. The thickness of the integument in most cats apparently prevents the penetration of this substance in the majority of cases.

Hexadienol applied to the human skin produced sweating in all cases that came under our observation. The onset of sweating varies from five to twenty-five minutes under identical conditions at average room temperature. Although no quantitative measurements were made, it may be stated that the amount of perspiration observed following hexadienol inunction is greater in high than in low temperatures. This is not surprising since sweating is favored by heat applied locally to the secretory nerve endings, for these respond to stimulation more actively if the skin is warm than when it is cold; in fact, cooling of an extremity can entirely prevent any response to stimulation (3). Following the application of 1 Gm. of hexadienol to a skin area two inches square the sweating may last for twelve hours. The sweating is not terminated by washing the skin with water, but is easily stopped by soap and water followed by alcohol.

B. Pharmacological Analysis of Hexadienol Diaphoresis.—In twelve cats in which hexadienol was given intravenously in doses mentioned above, the foot pad sweating was prevented or abolished in every case by intravenous injection of 0.1 mg. of atropine sulfate. The injection of a total dose of 0.2 cc. of a 1% solution of atropine sulfate into the foot pad likewise prevented or abolished the sweating produced either by intravenous or by local injection of hexadienol.

In six human subjects the sweating produced by local application of hexadienol was prevented in every case (*a*) by oral administration of 3 cc. of tincture of belladonna (Sharp and Dohme, U. S. P. X) given one hour prior to the dermal application of hexadienol, (*b*) by intracutaneous injection of 0.5 cc. of atropine sulfate solution (1:100,000) in the area on which the hexadienol was to be applied. In the same subject atropinization also terminated the sweating already induced by hexadienol, in the first instance after a delay up to one hour and in the second instance within a few minutes.

Following the method of Coon and Rothman (4) an attempt was made to investigate whether hexadienol sweating is due to a direct muscarine-like or a nicotine-like action.

In four cats the intravenous administration of 3 mg. of nicotine salicylate by vein did not prevent sweating induced either by intravenous injection of hexadienol or by injection of hexadienol into the foot pads. The injection of 0.5 cc. of a 1:200 solution of nicotine salicylate into the foot pad had no effect on the sweating induced either by intravenous or local injection of hexadienol.

In six human subjects a large area of the skin of the forearm was anesthetized by a subcutaneous injection of a 2% procaine hydrochloride solution. Immediately following the onset of numbness, hexadienol was applied to the anesthetized parts. Sweating occurred as usual on the anesthetized skin. Curiously, in two subjects the sweating was more pronounced on the anesthetized parts than on the surrounding area not affected by procaine. In all these subjects the intracutaneous injection of atropine sulfate immediately terminated the sweating.

C. The Use of Hexadienol in a Modified Minor's Technique.—Hexadienol diaphoresis may be successfully visualized for photographic diagnostic purposes by a modified Minor's technique (5). One gram of resublimed iodine was dissolved in 200 cc. of absolute alcohol and to this solution 300 Gm. of hexadienol was added. After stirring for about five minutes, 80 Gm. of fine soluble starch powder was added to the preparation which was stirred again for a sufficient length of time to obtain an approximately even distribution of the starch in the mix-

[1] Hexadienol, under the registered trademark name "hexene-ol," was supplied by the Consolidated Chemical Company, Cleveland, O.

[2] All doses used are expressed, unless stated otherwise, in mg. or Gm. per Kg. of body weight; for the sake of brevity the last phrase is omitted.

ture. The finished preparation has a light yellow color and when applied to the human skin the sweat which it produces imparts a dark bluish-black color to the skin. These dark spots are at first few in number, but later become more and more numerous and finally the whole area darkens. The moisture in the atmosphere has no effect on the yellow color of the preparation for when the above was applied to rabbit's skin or smeared over inanimate surfaces, no color change was observed. This preparation can be successfully used to demonstrate the presence of nonfunctioning sweat glands over the human skin. Clinical studies in which this diagnostic agent was employed in the study of the diseases or injuries of the sympathetic nervous system, are in progress.

D. Acute and Subacute Toxicity of Hexadienol.—It was deemed advisable to study the toxicity of hexadienol to guard against possible injurious effects that may follow the clinical use of this compound. Hexadienol was administered to rabbits either by subcutaneous injection or by application on completely denuded skin areas. The results of these studies may be briefly summarized by stating that each of twelve rabbits receiving a dose of 2 Gm. and each of twelve rabbits receiving 5 Gm. of hexadienol by the hypodermic route survived without symptoms. Ten rabbits receiving 10 Gm. of hexadienol survived and two died. Each of twelve rabbits received a subcutaneous injection of 2 Gm. of hexadienol daily for a period of eight weeks. During that time interval none of these rabbits showed any visible disturbance or signs of irritation. They showed no anorexia and lost no weight. Daily application of hexadienol on denuded skin areas of white rabbits did not interfere with wound healing and produced no visible injury. The denuded areas were one inch square, and about 1 Gm. of hexadienol was applied daily until complete healing occurred, or for about three weeks.

DISCUSSION

Hexadienol is the first substance known to the writer that induces sweating following local application to the skin. It is a sudorific ointment. The pharmacological study of the substance indicates that it acts upon the cholinergic transmittors at the effector since its action is blocked by atropine. It has no nicotinic action since the sweat response is not abolished by paralytic doses of nicotine or by infiltration of the human skin with procaine. Certainly there is no central action or sudomotor axone reflex involved. Hexadienol stimulates cholinergic sudomotor effectors but even massive doses do not appear to produce any observable parasympathetic stimulation with the possible exception of occasional salivation. It is not, in any sense, a parasympathetic stimulant. Its pharmacological action is curiously confined to the sudomotor effectors. Due to its lack of toxicity, emollient property and absence of systemic cholinergic effects, it is well suited for clinical use either as a diaphoretic or as a diagnostic agent. The simple device of terminating its action by an alcohol rub adds to the safety and convenience of its use.

It is a remarkable fact that lesions of postganglionic nerves to the periphery cause local anhydrosis of the skin (6, 7) and that cholinergic drugs fail to produce sweating in such denervated areas. It appears to be definitely established (8) that following preganglionic sympathectomy, pilocarpine will induce sweating indefinitely whereas after postganglionic sympathectomy, pilocarpine will fail to cause diaphoresis two months or longer after the operation. In other words, the sweating test, induced by parasympathetic drugs, will validly demonstrate anhydrotic areas due to sympathetic degenerations only when at least two months have elapsed following severance of peripheral nerves. It remains to be determined if hexadienol will, in this respect, behave like pilocarpine.

Practical diagnostic methods for the analysis of sympathetic cutaneous innervation include vasomotor, pilomotor and sudomotor responses. According to the best authorities available (9) only the sweating response is free of objections since it can be observed over the entire surface of the body and minute differences and the chronological appearance of sweat can be visualized and photographed conveniently. Methods for visualization of perspiration were furnished by the Minor test (5) or by painting the skin with an alcoholic solution of an anhydrous salt of cobalt which turns red in the presence of water (sweat) (10).

Several methods of inducing sweating have been proposed and they include heat sweating, emotional sweating, sweat produced by gustatory stimulation, spinal reflex sweating under certain pathological conditions and, finally, sweating induced by subcutaneous injection of parasympathetic stimulant drugs, pilocarpine, acetylbetamethylcholine hydrochloride and furfuryltrimethylammonium iodide (11). The last named method is the one now used in diagnosis of lesions of peripheral nerves unhappily common in war causalties (11).

These diagnostic procedures utilizing drugs have two disadvantages: (*a*) their occasional failure to induce sweating, (*b*) unpleasant side actions including frequent desire to micturate, nausea, vomiting, salivation, tear secretion, rales and rhonchi. Although these side actions may be easily combatted with the administration of belladonna alkaloids, these physiological anti-

dotes also interfere with the successful completion of the diagnostic test.

Since hexadienol does not possess any of these disadvantages, it is recommended that it should be given an extensive clinical trial as a diaphoretic agent and the iodized hexadienol-starch mixture be used in the mapping out and photographic visualization of the distribution of functioning sweat glands in the skin.

SUMMARY

Hexadienol produces sweating upon systemic and local administration in cats. It also produces sweating following local application to the human skin.

Hexadienol sweating can be easily terminated by washing the skin with alcohol.

Atropine prevents and abolishes this sweating both in cats and human subjects. Nicotine and procaine anesthesia have no effect on hexadienol diaphoresis. Hexadienol diaphoresis may be classed as a muscarine-like and not a nicotine-like action.

Hexadienol has no generalized parasympathomimetic activity.

Hexadienol is recommended for supplanting pilocarpine and other general parasympathetic stimulants as a sudorific and as a diagnostic agent in localizing anhydrotic areas in the skin. An iodized hexadienol-starch mixture is described as a new modification of the Minor test.

Hexadienol has a very low acute and subacute toxicity in rabbits and does not irritate the skin.

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Book Review

The Avitaminoses, by WALTER H. EDDY and GILBERT DALLDORF. The Williams and Wilkins Company, Baltimore Third Edition, 1944. xi + 438 pp. 15 x 23 cm. Price, $4.50.

Fifteen years have seen the vitamins rise from a state of biological curiosity and mystery to a position of nutritional and medical prominence surpassed by no other group of physiological agents. It has been the element of mystery which has encouraged the abuse of the vitamins to "cure" alleged maladies far beyond their physiological limitations. To aid the physician and the Pharmacist in culling the literature in this field, Doctors Eddy and Dalldorf, an excellent team of biochemist and pathologist, have written this third edition of *The Avitaminoses*.

This authoritative book is divided into three sections: The Vitamins, The Avitaminoses, and Technical Methods, Vitamin Assays and Vitamin Values. The first portion of 128 pages deals with the chemical nature and physiological function of the individual vitamins. This section has been brought up to date both from the standpoint of pure chemistry and the role the vitamins play in tissue metabolism. Of special value are the tabulations found in Chapter IV: minimum Food and Drug Standards for label control, recommended dietary allowances of the Food and Nutrition Board of the National Research Council, and special tests for vitamin deficiency.

Part two deals largely in pathological findings associated with the individual vitamin deficiencies. Additional chapters on the place of vitamin therapy in infectious diseases and medical care of nutritional failure are noteworthy for their sane viewpoint.

The laboratory worker will find the third section on methods of much help. Although in outline only, this section gives a careful evaluation of laboratory techniques. The documentation is authoritative and complete. In the section on vitamin C it is unfortunate that Dr. Musulin's name was misspelled and bichloracetic acid instead of trichloracetic acid is listed as the stabilizing agent.

As evidence of the completeness of documentation, the 32 pages of references conveniently arranged to correspond to the individual chapters might be cited.

No library in any of the many related fields of Pharmacy can afford to be without this reliable and authoritative volume.—MELVIN W. GREEN.